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(54) Title: APPARATUS AND METHOD FOR TRAPPING BEAD BASED REAGENTS WITHIN MICROFLUIDIC ANALYSIS SYSTEMS

(57) Abstract: The present invention provides an on-chip packed reactor bed design that allows for an effective exchange of packing materials such as beads at a miniaturized level. In accordance with the present invention, there is provided a method of concentrating an analyte within a microfluidic analysis system, comprising the steps of: a) providing a main channel having a trapping zone suitable for trapping packing material; b) providing a slurry of a reagent treated packing material prepared in a solution having a predetermined composition of a solvent; c) inducing a flow of said packing material into said trapping zone through a flow channel connected to said trapping zone so as to load said trapping zone and form a packed bed of said packing material; d) and flowing a sample containing analytes through said packed bed, said reagent acting to concentrate at least some of said analytes within said trapping zone. The present invention extends the function of microfluidic analysis systems to new applications including on-chip solid phase extraction (SPE) and on-chip capillary electrochromatography (CEC). The design can be further extended to include integrated packed bed immuno- or enzyme reactors.



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**Title: Apparatus and Method for Trapping Bead Based Reagents  
Within Microfluidic Analysis Systems**

**FIELD OF THE INVENTION**

5 The present invention relates generally to microfluidic analysis systems, and more specifically to micro-Total Analysis Systems ( $\mu$ -TAS), for performing liquid phase analysis at a miniaturized level.

**BACKGROUND OF THE INVENTION**

Recent developments in the field of micro-Total Analysis Systems ( $\mu$ -TAS) have led to systems that perform chemical reactions, separation and detection at a miniaturized level on a single microchip [see, for example, Harrison, D. J.; Fluri, K.; Seiler, K.; Fan, Z.; Effenhauser, C. S.; and Manz, A., *Science* 1993, 261, 895-897. Harrison, D.J.; and van den Berg, E.; Eds., *Micro Total Analysis Systems '98, Proceedings of the  $\mu$ TAS '98 Workshop* (Kluwer: Dordrecht, 1998). Coyle, C. L.; Tang, T.; Chiem, N.; and Harrison, D. J., *Electrophoresis* 1997, 18, 1733-1741].

Most prior art microfluidic devices are based on conventional open tubular flow designs and solution phase reagents. While the functionality of these devices has continued to increase, one key feature that is presently lacking in these prior art devices is the ability to effectively incorporate on-chip packed reactor beds, for introduction of packing materials with immobilized reagents or stationary phases. While a few attempts have been made to employ packed reactor beds in some prior art designs, the difficulty of packing portions of a complex microfluidic manifold with packing material (such as microscopic beads) has so far hindered the effective utilization of these reagent delivery vehicles within microfluidic devices. (The difficulty of packing has been well recognized by practitioners in the field. See, for example, Ericson, C; Holm, J.; Ericson, T.; and Hjertén, S., *Analytical Chemistry*.)

In one prior art example, a packed bed chromatographic device with a bead trapping frit was fabricated in a silicon substrate [Ocvirk,

G., Verpoorte, E., Manz, A., Grasserbauer, M., and Widmer, H. M. *Analytical Methods and Instrumentation* 1995, 2, 74-82]. However, the packing material in this prior art design could not be readily packed or exchanged, thus limiting its utility.

- 5               Several authors have also described the difficulties associated with reproducibly fabricating frits for retaining packing material in conventional capillaries [Boughtflower, R.J.; Underwood, T.; Paterson, C. J. *Chromatographia* 1995, 40, 329-335. Van den Bosch, S. E.; Heemstra, S.; Kraak, J. C.; Poppe, H. J. *Chromatogr. A* 1996, 755, 165-177. Colon, L. A.; Reynolds, K. J.; Alicea-Maldonado, R.; Fermier, A. M. *Electrophoresis* 1997, 18, 2162-2174. Majors, R. E. *LC-GC* 1998, 16, 96-110.]. The frits used in conventional systems are prepared using time and labor intensive procedures, the most commonly used method involving the use of pure silica gel, wetted down with aqueous sodium silicate. The frit is made by first tapping a capillary end into a paste made from silica and aqueous sodium silicate. The resulting plug of silica is then heated to make a frit. Current construction methods do not produce high yields of useable frits.

- Furthermore, using frits produced by prior art methods of construction often leads to the formation of undesirable bubbles. [Altria, K.D.; Smith, N.W.; and Turnbull, C.H., *Chromatographia*, 46 (1997) 664. Majors, R.E., *LC-GC*, 16 (1998) 96.] Bubbles cause discontinuity within a column, hindering solution flow and ultimately preventing separation from occurring. The bubbles are thought to arise from a change in electroosmotic flow (EOF) velocity caused by moving from a bead trapping frit into an open capillary. The formation of bubbles, which have been observed to increase at higher voltages, also limits the amount of voltage that can be applied across the capillary, thereby limiting column length, separation efficiency, and speed of analysis.

- Developing a functional on-chip packed reactor bed design which overcomes the limitations in the prior art would significantly enhance the range of the microfluidic toolbox and extend the number of applications of such devices.

### SUMMARY OF THE INVENTION

Generally, the present invention provides an on-chip packed reactor bed design using one or more weir structures that allow for an effective exchange of packing materials (beads for example) at a miniaturized level. The present invention extends the function of microfluidic analysis systems to new applications. For example, the packed reactor bed formed according to the present invention allows on-chip solid phase extraction (SPE) and on-chip capillary electrochromatography (CEC), as explained in detail further below. The design can be further extended to include, for example, integrated packed bed immuno- or enzyme reactors.

In a first aspect, the present invention provides a method of treating a sample within a microfluidic analysis system, comprising the steps of:

- a) providing a main channel having a trapping zone suitable for trapping packing material;
- b) providing a slurry of a reagent treated packing material prepared in a solution having a predetermined composition of a solvent;
- c) inducing a flow of said packing material into said trapping zone through a flow channel connected to said trapping zone so as to load said trapping zone and form a packed bed of said packing material;
- d) flowing a sample containing analytes through said packed bed, said reagent treating the sample, whereby the sample leaving the trapping zone has an altered analyte composition.

In one embodiment, the method further comprises the step of:

- e) adjusting the composition of the solvent, so as to affect the aggregation of said packing material and the stabilization of the packed bed.

In another embodiment, step b) comprises providing packing

In another embodiment, said porous beads are selected to have a diameter in the range from about 0.7 to about 10.0  $\mu\text{m}$ .

In yet another embodiment, said porous beads are selected to have a diameter in the range from about 1.5 to about 4.0  $\mu\text{m}$ .

- 5           In another embodiment, said solvent is acetonitrile, and step e) comprises adjusting the concentration level to less than about 50% to stabilize the packed bed. ·

- In another embodiment, said solvent is acetonitrile, and step e) comprises adjusting the concentration level to less than about 30% to  
10 stabilize the packed bed.

In another embodiment, the method further includes the steps of adjusting the concentration level to above 50% to destabilize the packed bed, and reversing the flow in step c) so as to unload said trapping zone.

- 15           In another embodiment, the method further includes the step of repeating step c) so as to reload said trapping zone, and readjusting the concentration level to restabilize the packed bed.

In yet another embodiment, the method further comprises the steps of:

- 20           before step c), adding a neutral surfactant to said packing material so as to inhibit aggregation; and  
          after step c), removing the neutral surfactant to promote aggregation.

In another embodiment, the method further comprising the steps of:

- 25           after step c) introducing a polymerizable agent into the flow channel and polymerizing said agent, so as to stabilize the packed bed.

In yet another embodiment, step d) comprises applying a fluid force to induce the flow of said packing material.

- 30           In another embodiment, said packing material comprises at least some electrically charged particles and step d) comprises applying a voltage potential to induce the flow of said packing material.

In another embodiment, said packing material comprises at least some particles susceptible to a magnetic field and step d) comprises applying a magnetic field to induce the flow of said packing material.

- 5 In another embodiment, the method further includes the step of providing a hook structure at the connection point between said flow channel and said trapping zone, so as to prevent direct line-of-sight entry of said packing material, thereby to promote even packing.

In another aspect, the present invention provides a method of trapping bead based reagents within a microfluidic analysis system,  
10 comprising the steps of:

- a) providing a main channel having a trapping zone suitable for trapping bead based packing material;
- b) providing a slurry of a reagent treated beads prepared in a solution having a predetermined composition of a  
15 solvent;
- c) inducing a flow of said beads into said trapping zone through a flow channel connected to said trapping zone so as to load said trapping zone and form a packed bed of said beads; and
- 20 d) adjusting the composition of the solvent, so as to affect the aggregation of said packing material and the stabilization of the packed bed.

In a further aspect, the present invention provides a microfluidic analysis system, comprising:

- 25 a) a substantially planar substrate having an upper surface;
- b) at least one main channel formed into said upper surface, said main channel having first and second ends and a defined direction of flow in use;
- c) a cover plate arranged over said planar substrate, said  
30 cover plate closing off said channel from above; and
- d) a first weir formed across said main channel and between said first and second ends of said channel, said first weir

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providing at least one flow gap to allow, in use, at least some fluid to flow past said first weir while trapping packing material having constituent particles that are generally larger than said flow gap.

5 In one embodiment, the system further comprises at least one side channel formed into the upper surface of said planar substrate, said side channel being connected at a first end to said main channel at a location upstream from said first weir, and at a second end to a reservoir, said side channel providing a higher flow resistance than said main  
10 channel.

In another embodiment, the system further comprises a second weir located upstream from said connected first end of said side channel, said first and second weirs forming a chamber therebetween, said second weir providing at least one flow gap to allow, in use, at least some fluid to  
15 flow past said second weir while trapping said packing material within said chamber.

In another embodiment, each side channel connection to said main channel is provided with a hook structure curved to one side whereby, in use, packing material is sprayed into said chamber to facilitate  
20 even packing.

In another embodiment, said hook structure at least partially obstructs direct line-of-sight entry of packing material from said side channel into said chamber and forms a chamber mouth to one side of said hook structure.

25 In another embodiment, said flow gaps comprise a generally uniform gap between said cover plate and the top of said weirs.

In yet another embodiment, said flow gaps comprise a plurality of substantially vertical gaps in said weirs.

In another embodiment, said system is formed entirely on a  
30 single microfluidic chip.

In another embodiment, the present invention provides a method comprising, providing a non-conductive substrate and effecting

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an electrokinetic flow by applying a relatively high voltage at said second end of said main channel and at said reservoir, said reservoir containing packing material, and providing a relatively low voltage at said first end of said main channel, so that packing material flows from said reservoir into said main channel and is trapped against said first weir.

In one embodiment, the packing material is removed from said main channel by providing a relatively high voltage at said first and second ends of said main channel while providing a relatively low voltage at said reservoir.

In another aspect, the present invention provides a method comprising, effecting a pressure driven flow by providing a relatively high pressure at said second end of said main channel and at said reservoir, said reservoir containing packing material, and providing a relatively low pressure at said first end of said main channel, so that packing material flows from said reservoir into said main channel and is trapped against said first weir.

In one embodiment, the packing material is removed from the said main channel by providing relatively high pressure at said first and second ends of said main channel while providing relatively low pressure at said reservoir.

In another embodiment, the method comprises providing a non-conductive substrate and effecting an electrokinetic flow by applying a relatively high voltage at said reservoir, said reservoir containing packing material, and providing relatively low voltages at said first and second ends of said main channel, so that packing material flows from said reservoir into said chamber and is trapped by said first and second weirs.

In another embodiment, the packing material is removed from the chamber by reversing said electrokinetic flow.

In another aspect, the present invention provides a method of packing the chamber in a microfluidic analysis system comprising, effecting a pressure driven flow by providing a relatively high pressure at said reservoir, said reservoir containing packing material, and providing



relatively low pressure at said first and second main reservoirs, whereby, packing material flows from said packing material reservoir into said chamber and is trapped by said first and second weirs.

In another embodiment, the packing material may be removed  
5 from the chamber by reversing said pressure driven flow.

In another embodiment, the method comprises providing magnetically charged packing material, and effecting a magnetically driven flow by providing a magnetically attractive force in the chamber, whereby, the packing material enters the chamber and is trapped by said first and  
10 second weirs.

In another embodiment, the packing material may be removed from the chamber by reversing said magnetic force in said chamber.

In any of the above embodiments, the packing material may comprise porous beads.

In another embodiment, the beads may be generally spheroid.

In another embodiment, the beads are initially suspended in a buffer solution.

In another embodiment, the buffer solution is an organic solvent miscible with water.

In another embodiment, the organic solvent is acetonitrile  
20 with a concentration level of up to 50%.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

For a better understanding of the present invention, and by  
25 way of example, reference will now be made to the accompanying drawings, which show preferred embodiments of the present invention in which:

Figure 1A shows a top plan view of a microfluidic device according to the present invention;

30 Figure 1B shows an enlarged perspective view of a chamber in which packing materials (such as beads) are trapped;

Figure 2A shows a cross-sectional view of the chamber shown

in Figure 1B taken along line A-A, and further shows packing material (beads) which are packed into the chamber and which are retained by a cover plate;

Figures 2B and 2C show a side view and end view, respectively, of an alternative embodiment of a weir according to the present invention;

Figure 3A shows an initial stage of packing material (beads) being packed into the chamber shown in Figures 1B and 2A;

Figure 3B shows the chamber of Figure 3A after it has been completely filled with packing material (beads);

Figure 4A shows an early stage of preconcentration of a 1.0 nM BODIPY solution at the weir/bed interface near the top of Figure 4A;

Figure 4B shows a later stage of preconcentration of a 1.0 nM BODIPY solution at the weir/bed interface near the top of Figure 4B;

Figure 5 shows a plot of fluorescence intensity vs. time, showing fluorescence of a first 1.0 nM BODIPY sample during loading, followed by a buffer flush, and then preconcentrated BODIPY during elution with acetonitrile (ACN);

Figure 6 shows an electrochromatogram of BODIPY and fluorescein, showing different steps of the separation including load, flush, and elution;

Figures 7A-7D show electrochromatograms of BODIPY and fluorescein with different concentrations of acetonitrile in the mobile phase, specifically at: (a) 30%; (b) 22%; (c) 15%; and (d) 10%;

Figure 8A-8C show top plan views of alternative embodiments of a microfluidic device according to the present invention;

Figure 9 shows a top plan view of a microfluidic device according to the present invention having multiple packed chambers;

Figure 10 shows a schematic view of a microfluidic device according to the present invention being used in conjunction with a mass spectrometer;

Figure 11 shows a graph plotting the fluorescence intensity of

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theophylline against time, as it saturates a packed bed;

Figure 12 shows theophylline being eluted from packed bed in a relatively narrow band; and

Figure 13 shows each successive trial resulting in lower light generated from the CL reaction.

### **DETAILED DESCRIPTION OF THE INVENTION**

As explained above, the present invention is designed to provide a convenient system and method of trapping packing materials (such as beads) on-chip, and of effectively packing and unpacking the trapping zones, to provide a functional on-chip packed reactor bed which significantly extends the number of applications of microfluidic analysis devices.

One such extended application facilitated by the present invention is on-chip sample preconcentration by solid phase extraction (SPE). In microfluidic analysis, SPE is often required to overcome detection limit problems, or to eliminate a potential interferent. To date, preconcentration within microchips has been performed by sample stacking using "isoelectric focusing" [Jacobson, S. C. and Ramsey, M. *Electrophoresis* 1995, 16, 481-486]. Advantageously, unlike sample stacking, SPE can be made selective for a particular analyte and does not require precise control of buffer concentrations. For SPE the amount of preconcentration is limited by the preconcentration time, which makes it more flexible than sample stacking. The SPE of an analyte can be beneficial not only for analyte preconcentration, but also for removing other impurities or changing solvent conditions. While the coupling of SPE with microfluidic devices has been accomplished [Figeys, D. and Aebersold, R. *Anal. Chem.* 1998, 70, 3721-3727], the SPE component in these prior art devices have been made in a capillary or similar cartridge external to the chip, thus resulting in a more complex and more expensive system. The present invention is designed to overcome this prior art limitation by facilitating an on-chip SPE component.

As realized by the present inventors, an integrated, on-chip SPE component is ultimately easier to manufacture, does not require low dead volume coupling to the chip, and eliminates sample handling losses or contamination problems arising from the off-chip sample manipulation required in the prior art. It is anticipated that routine incorporation of SPE onto a chip, as facilitated by the present invention, will reduce problems with on-chip detection limits and will improve the range of sample preparation steps which can be integrated.

Another extended application facilitated by the present invention is on-chip capillary electrochromatography (CEC). CEC has recently received significant attention due to the fact that it combines the separation power of both liquid chromatography and capillary electrophoresis. To date the difficulty associated with packing chromatographic material within devices has focused most previous chromatographic efforts upon prior art open channel methods [Manz, A., Miyahara, Y., Miura, J., Watanabe, Y., Miyagi and H. Sato, K., *Sens. Actuators* 1990, B1, 249-255; Jacobson, S. C., Hergenröder, R., Koutny, L. B. and Ramsey, J. M. *Anal. Chem.* 1994, 66, 2369-2373; Kutter, J. P., Jacobson, S. C., Matsubara, N. and Ramsey, J. M. *Anal. Chem.* 1998, 70, 3291-3297; He, B., Tait, N. and Regnier, F. *Anal. Chem.* 1998, 70, 3790-3797]. In the prior art, open channel method devices with channel widths of 2  $\mu\text{m}$  or less were required to improve mobile-phase transfer in open columns leading to other practical considerations such as clogging and a short path length for detection. There were also problems with the reproducibility and the cost of stationary phase coating in such structures.

As realized by the inventors, on-chip packed bed chromatography according to the present invention has the benefit of providing low mobile-phase mass transfer, and makes available a wide variety of stationary phases. In this case, the use of an off-chip prepared stationary phase offers the advantage that it eliminates the need for coating the chip and allows for optimization of the stationary phase preparation.

Yet another extended application facilitated by the present invention is providing on-chip bead-based immunoassay and enzyme based assays. These applications are described further below.

#### EXAMPLE

- 5 To illustrate the present invention by way of example, the inventors conducted a series of experiments, which are described here.

#### Chip Design

- 10 Figures 1A and 1B show a microfluidic device 10 as used in these experiments. The device 10 comprises a main channel 11 formed into the top surface of a substrate 8, and the main channel 11 is separated by a chamber 4, also formed into the substrate 8. Two branches of the main channel 11, as separated by the chamber 4, are further identified as main reservoirs 1 and 2. The chamber 4 is connected to a packing material reservoir 3 by a narrow side channel 5. The packing material reservoir and the narrow side channel 5 are also formed into the substrate 8. Figure 1B shows an enlarged image of the chamber 4 obtained with a scanning electron microscope (Jeol X-Vision JSM6301FXV, Peabody, MA). The chamber 4 is formed by providing two weirs 6, 7 formed across the main channel 11 at a relatively narrow portion of the main channel 11 (Figure 1A). As can be seen from Figure 1B, the weirs 6, 7 are not as high as the main channel 11 is deep, so that some fluid is allowed to flow over the weirs 6, 7 as explained below. The device 10 was prepared in Corning 0211 glass by the Alberta Microelectronic Corporation (Edmonton, AB), using known chemical etching procedures [Fan, Z. H.; Harrison, D. J. *Anal. Chem.* 1994, 66, 177-184]. It is noted that this substrate material is non-conductive, but if other than electrokinetic forces are being used (as detailed further below), then the substrate material may be semiconducting or conducting. Two photomasks were required to create device 10: a first photomask was used to etch the tops of the weirs 6, 7 to a depth of approximately 1  $\mu\text{m}$ ; and a second photomask was used to etch
- 15  
20  
25  
30

the channels 5, 11 to a depth of approximately 10  $\mu\text{m}$ .

Figure 2A shows a cross-sectional view of the weirs 6, 7 which are not as high as the channel 11 (main reservoirs 1, 2) is deep, and thus small flow gaps 14, 15 are provided between the top of the weirs 6, 7 and a cover plate 9 (not shown in Figure 1A or 1B) which is placed on top of the substrate 8, thereby closing off the chamber 4, channels 5, 11 and reservoirs 1, 2, 3. As can be seen in Figure 2A, the beads 12 are generally larger than the flow gaps 14, 15 and therefore cannot escape from the chamber 4.

Figures 2B and 2C show a side view and an end view, respectively, of an alternative embodiment of a weir 6' in which substantially vertical notches 6'' are provided so that the weir 6' provides less flow impedance. The vertical notches 6'' should be narrow enough that no beads can pass through them (i.e. they should be at least about 10% smaller than the smallest bead diameter).

## 15 **Solutions and Reagents**

Various solutions and reagents were used in these experiments. Acetonitrile (BDH, Toronto, ON) was filtered through a 0.45  $\mu\text{m}$  Nylon-6,6 filter (Altech, Deerfield, IL) prior to use. Otherwise, the acetonitrile was used as received, with no added electrolyte. Also, 50 mM potassium phosphate (pH 7.0) and ammonium acetate (pH 8.5) buffers were prepared in ultra-pure water (Millipore Canada, Mississauga, ON). A 1:1 (v/v) mixture of acetonitrile and buffer was prepared. A stock solution of 0.10 mM, 4,4'-difluoro 1,3,5,7,8-penta-methyl-4-bora-3a,4a-diaza-s-indacene, BODIPY 493/503 (Molecular Probes, Eugene, OR) was prepared in HPLC grade methanol (Fisher, Fair Lawn, NJ). A 1 mM stock solution of fluorescein di-sodium salt (Sigma) was prepared in phosphate buffer. Both stock solutions were then diluted in the 50 mM phosphate and 50 mM ammonium acetate buffers to give 1.0  $\mu\text{M}$  solutions, which were then diluted to 1.0 nM. This 1.0 nM solution served as the sample for preconcentration and electrochromatography. All aqueous (buffer and sample) solutions were filtered through a cellulose

acetate syringe filter (0.2  $\mu\text{m}$  pore size) (Nalgene, Rochester, NY) prior to use.

### Packing Material

One suitable packing material used in these experiments  
5 comprised a reverse-phase chromatographic stationary resin. The resin was Spherisorb ODS1 (Phase Separations, Flintshire, UK), a porous C-18 resin whose particles ranged from 1.5 to 4.0  $\mu\text{m}$  in diameter, as determined by scanning electron microscopy (ODS beads 12). A slurry of approximately 0.003 g/mL of ODS1 was prepared in acetonitrile. This slurry was used to  
10 supply the packing material reservoir 3, to subsequently pack the chamber 4.

Certain solvent and additive combinations were found to help the packing material stay in the packed chambers. For example, if ODS beads are introduced in acetonitrile they flow readily, while  
15 subsequently switching to an aqueous or predominately aqueous solvent causes the beads to aggregate and become trapped within the chamber. With ODS beads up to 30% acetonitrile could be present in the aqueous solution without disrupting the aggregation observed to the point of destabilizing the packed bed. Up to 50% acetonitrile could be present with  
20 only modest loss in aggregation and weak destabilization of the bed. As another example protein G or protein A coated beads formed aggregates in aqueous solution, which made it hard to introduce them into the trapping zone. However, the addition of a neutral surfactant such as Tween 20 or Brij 35 (both are trademarks) prevented such aggregation and allowed the  
25 beads to be introduced. Conversely, subsequent removal of the surfactant from the aqueous solvent resulted in aggregation and enhanced stability of the trapped bed. The following trend was observed: when using non-polar or partially non-polar bead phases (for example, ODS and protein coated beads) lowering the surface tension of the solvent from that of water or  
30 buffered water, by the addition of organic additives such as organic solvents or surfactants, reduced the tendency to aggregate. Conversely

- 15 -

- reducing or eliminating materials with lower surface tension from aqueous solution increased the tendency to lock the beads in place on the bed, creating a "solvent lock" method to enhance bead trapping within these devices. Other organic solvents other than acetonitrile, miscible with water may also be used for these purposes, such as methanol, ethanol, dimethylsulfoxide, propylene carbonate, etc. Charged surfactants may also be used instead of neutral surfactants, so long as they are compatible with the proteins that may be present on the beads or in the sample.
- Magnetic beads used for magnetic packing may comprise Abebaw - protein "A" coated beads: composition 36-40% magnetite dispersed within a copolymer matrix consisting of styrene and divinyl benzene (Prozyme, California) Also, Guifeng - oligo (dT)<sub>25</sub> coated beads may be used for the isolation of mRNA. The beads have an even dispersion of magnetic material (Fe<sub>2</sub>O<sub>3</sub> and Fe<sub>3</sub>O<sub>4</sub>) through out the bead. The beads are coated with a polystyrene which encases the magnetic material (Dynal, Oslo, Norway).

### Instrumentation

- Various instruments were used in conducting the present experiments. As these instruments and their operation are well known to those skilled in the art, only a brief description is provided, and the instruments are not shown in the figures.

- A power supply and relay system used to control the electrophoretic voltages necessary for bead packing and all liquid handling on-chip has been described previously [Fluri, K., Fitzpatrick, G., Chiem, N. and Harrison, D.J. *Anal. Chem.* 1996, 68, 4285-4290]. LabVIEW programs (National Instruments, Austin, TX), were written for computer control of the voltage system and for data acquisition.

- A laser-induced fluorescence detection system used in this experiment consisted of a 488 nm argon ion laser (Uniphase, San Jose, CA), operated at 4.0 mW, and associated focusing optics [Manz, A., Miyahara, Y.,



Miura, J., Watanabe, Y., Miyagi, H. and Sato, K. *Sens. Actuators* 1990, B1, 249-255] (Melles Griot, Irvine, CA). Fluorescence emitted from the BODIPY sample (as described above) was collected by a 25X, 0.35 NA microscope objective (Leitz Wetzlar, Germany). The images were observed with a  
5 SONY CCD-IRIS camera. Alternatively a 530 nm emission filter and a photo multiplier tube (PMT) (R1477, Hamamatsu, Bridgewater, NJ) were used as a detector positioned so that the narrow channel 5 between the chamber 4 and packing material reservoir 3 could be monitored. Data was collected from the section of main channel 11 just next to the chamber 4.  
10 The weir 6 was just out of the field of view. The PMT was biased at 530 V while the PMT signal was amplified, filtered (25 Hz Butterworth) and sampled at a frequency of 50 Hz.

The fluorescence of the buffer, acetonitrile, and 1.0 nM BODIPY in both buffer and acetonitrile was measured using a Shimadzu  
15 RF-5301PC Spectrofluorophotometer.

While specific models and manufacturers have been provided for various instrumentation described above, it will be understood by those skilled in the art that any suitable, functional equivalent may be used.

## 20 Chip Operation

Referring back to Figures 1A and 1B, the narrow side channel 5 leading into the chamber 4 from packing material reservoir 3 was used to direct stationary phase packing material into the chamber 4 using electrokinetic pumping [Yan, C., U. S. Patent 5 453 163, 1995; Knox, J. H.  
25 and Grant, I. H. *Chromatographia* 1991, 32, 317-328]. As mentioned above, the substrate 8 is non-conductive, which allows packing of the beads 12 using the electrokinetic pumping method.

The device 10 was not conditioned with any aqueous solutions prior to use. The chamber 4, channels 5, 11, and reservoirs 1, 2, 3  
30 were first filled with acetonitrile. The chamber 4 was packed with ODS beads 12 (Figure 2) by replacing the acetonitrile in packing material

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reservoir 3 with the ODS/acetonitrile slurry (described above), then applying positive high voltage at packing material reservoir 3 while holding main reservoirs 1 and 2 at ground. The voltage applied at packing material reservoir 3 was ramped from 200 V to 800 V over approximately 5 min to effect packing of chamber 4.

Once the chamber 4 was packed, a step gradient was performed to introduce aqueous solution to the main channel 11 and the ODS beads 12 in the chamber 4. A 1:1 (v/v) mixture of acetonitrile and buffer was placed in reservoirs 1 and 2. Acetonitrile replaced the slurry in packing material reservoir 3. A voltage was then applied to main reservoir 1 and was ramped from 200 V to 800 V, with packing material reservoir 3 biased at 400 V and main reservoir 2 grounded. After 2 to 5 min at 800 V, the acetonitrile/buffer mixture in reservoirs 1 and 2 was replaced with buffer, and the same voltage program repeated. The chamber 4 was monitored visually to ensure that the acetonitrile was completely replaced by buffer and that the packing material (beads 12) did not shift or unpack during this procedure. (The beads 12 could be seen to agglomerate as the acetonitrile was expelled, and the index of refraction change at the water/acetonitrile interface was clearly visible.) The experiments conducted are described in further detail below.

### **Experimental Results and Discussion**

In order to conduct the experiments, it was necessary to pack the chamber 4 with packing material (beads 12), as shown in Figure 2A.

The narrow side channel 5 shown in figures 1A and 1B was made to be about 30 $\mu$ m wide to supply packing material (beads 12) to the chamber 4. A sample could then be delivered from reservoir 2 (the inlet channel), across the chamber 4 and on towards main reservoir 1 (the outlet channel). The volume of the chamber 4 was 330 pL, while the volume of the outlet and inlet channels was  $1.5 \times 10^{-7}$  L and  $4.1 \times 10^{-8}$  L, respectively. The main channel 11 had much lower flow resistance than the side channel 5, in spite of the weirs 6, 7, given their relatively wide widths (580

$\mu\text{m}$ , tapering to 300  $\mu\text{m}$  at the weirs) in comparison to the width of the narrow channel 5 (30 $\mu\text{m}$ ). The relative flow resistance in the device 10 was manipulated by the selection of the width dimensions for these channels 5, 11 in order to encourage flow between main reservoirs 1 and 2, rather than into the narrow bead introduction side channel 5 during sample loading and elution.

Reverse phase ODS beads 12 (as described previously) were used in the SPE device because of their extensive use for the chromatography of proteins, peptides and tryptic digests [Seifar, R. M.; Kok, W. T.; Kraak, J. C.; and Poppe, H. *Chromatographia*, 1997, 46, 131-136. Yan, C.; Dadoo, R.; Zhao, H.; Zare, R. N.; and Rakestraw, D. J., *Anal. Chem.* 1995, 67, 2026-2029.] as well as other applications of SPE and CEC [Nielsen, R. G.; Riggins, R. M.; Rickard, E. C. *J. Chromatogr.* 1989, 480, 393-401. Hancock, W. S.; Chloupek, R. C.; Kirkland, J. J.; Snyder, L. R. *J. Chromatogr. A* 1994, 686, 31-43.]. Electrokinetic packing of conventional capillaries has been described previously, [Yan, C.; U. S. Patent 5 453 163, 1995. Knox, J. H.; Grant, I. H. *Chromatographia* 1991, 32, 317-328.], and the inventors have adapted the method for the present invention.

As briefly explained earlier, the packing procedure involved applying a positive voltage (ramped from 200-800 V) to the packing material reservoir 3, while grounding main reservoirs 1 and 2. The applied voltage induced EOF to flow down the bead channel, carrying the beads into the cavity. An organic solvent was required to suspend the chromatographic beads 12 to prevent them from aggregating and plugging the narrow side channel 5. Studies have shown that capillaries filled with acetonitrile exhibit substantial electroosmotic flow [Wright, P. B.; Lister, A. S.; Dorsey, J. G. *Anal. Chem.* 1997, 69, 3251-3259. Lister, A. S.; Dorsey, J. G.; Burton, D. E. *J. High Resol. Chromatogr.* 1997, 20, 523-528. Schwer, C.; Kenndler, E. *Anal. Chem.* 1991, 63, 1801-1807. Salimi-Moosavi, H.; Cassidy, R. M. *Anal. Chem.* 1995, 67, 1067-1073.].

As shown in Figure 3A, at the early stages of packing, the beads 12 entering the chamber 4 contacted the weirs 6, 7 on either side of

the chamber 4. As explained earlier, the beads 12 are unable to traverse the weirs 6, 7 because the distance from the top of the weirs 6, 7 to the bottom of the cover plate 9 (approximately 1.0  $\mu\text{m}$ ) is less than the diameter of the individual particles of the ODS beads 12 (approximately 1.5 - 4.0  $\mu\text{m}$ ).

5           As shown in Figure 3B, the chamber 4 continued to pack until it was entirely filled with chromatographic material. As discussed earlier, the difficulties associated with reproducibly fabricating frits for retaining packing material is well known. Importantly, the weir design used in the present invention circumvented this problem, and the  
10       electrokinetic packing of the beads provided an even distribution of beads throughout the chamber with no observable voids. In fact, the use of weir structures may ultimately eliminate the need for on column frit fabrication.

          The weir design of the present invention allows electric fields  
15       to be applied across the trapping zone formed by two weirs, when filled with beads, in a range as high as 20,000 to 80,000 V/cm without bubble formation at the weir. Separations performed in devices with these weirs can use electric fields at least as high as 15,000 V/cm. The power dissipated across a weir can be as high as 3-7 W/m without the formation of bubbles.  
20       In contrast frits formed in conventional columns have at the best been reported to form bubbles at power dissipations above 0.6 W/m, and electric fields in the range of 150-600 V/cm are the best that have been reported without bubble formation.

          It is possible to couple an external capillary to a chip and  
25       allow the weir to be used as the trapping element for the beads packed within the external electrochromatography capillary. This can be accomplished using a low dead volume coupling, such as described by Bings et al. (N.H. Bings, C. Wang, C.D. Skinner, C.L. Colyer, P. Thibeault, D.J. Harrison, *Anal. Chem.* 71 (1999) 3292-3296.) In this way the chip based  
30       weir can replace the frits normally formed within external capillaries, and allow higher electric fields to be used, improving speed and separation efficiency.

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(It is noted here that it was also possible to pack the cavity by applying a vacuum at main reservoirs 1 and 2, although this was less convenient when electrokinetic flow was used for sample loading and elution.)

5 If for some reason the beads 12 did not pack as tightly as was desirable (as shown in Figures 2 and 3B) they were removed from the chamber 4 by simply reversing the voltages, and the packing procedure was then repeated. It is noted that once an aqueous solution was introduced to the chamber 4, the reverse-phase beads 12 tended to  
10 aggregate and were more difficult to remove. However, subsequent removal was accomplished by flushing the aqueous solution out with acetonitrile, using either EOF or vacuum, or a combination of the two. Advantageously, the ability to effectively remove the beads 12 from chamber 4 allowed used chromatographic beads to be refreshed, or a more  
15 applicable material to be substituted.

Significantly, a design utilizing a hook structure 13 at the chamber entrance (Figures 1B and 3A) yielded the most favorable results in packing, enabling the chamber 4 to be packed and remain so after removal or alteration of voltages or vacuum. As seen from the figures,  
20 the side channel 5 connects to the chamber 4 via a chamber mouth 4A in an asymmetric fashion, relative to the weirs 6, 7. Also, the hook structure 13 preferably obstructs direct line-of-sight entry of packing material from the side channel 5 into the chamber 4. Rather, the hook structure 13 forces packing material to enter the chamber 4 indirectly via the chamber mouth  
25 4A.

As explained earlier, during the packing step, the packing material reservoir 3 has a positive bias applied with reservoirs 1 and 2 grounded. The inventors believe that the hooked structure 13 causes electric field lines to follow a curved pathway into the cavity.  
30 Consequently, as the chromatographic beads 12 follow the electric field lines into the chamber mouth 4A they appear to be "sprayed" as if from a snow blower (Figure 3A), to become uniformly packed.

During the packing procedure the chamber 4 filled only to the beginning of the hook structure 13 (see Figure 3B). Once filled, the beads were observed to flow down the sides and up the middle of the narrow side channel 5 (toward packing material reservoir 3) mimicking the solvent back flow generated in a closed electrophoretic system [Shaw, D. J. *Introduction to Colloid and Surface Chemistry*, 3<sup>rd</sup> ed. Butterworths: London, 1980.]. In such a closed system, EOF is directed along the walls until it reaches the end of the chamber, where pressure causes the solution to reverse direction and flow back up the center of the bead introduction channel.

A key aspect of the hooked structure as shown is the asymmetric entrance into the trapping zone, which allows for better packing. A symmetric entrance means the entering beads can go to both weirs equally, which tends to lead to uneven or difficult packing. An asymmetric structure allows the beads to pack preferentially at one end of the trapping zone first and then build up in one direction from that location. The key role of the hook structure is to prevent line-of sight outflow from the trapping zone during use of the packed bed.

Chambers constructed without an asymmetry in the entrance were not observed to pack as well as asymmetric entry designs. In these cases, packing material tended to fill the corners furthest from the entrance, but no additional material would enter the chamber. The inventors believe that, due to its symmetric design, this type of chamber exhibits solvent back flow, after it has filled to a certain extent. That is, the partially filled chamber may resemble a closed or restricted system. Such an occurrence would preclude the filling of the symmetric chamber with beads and is consistent with previously observed behavior, as explained by Shaw. Such behavior may account for the ability to fill symmetric structures on some occasions but less readily on others. In contrast, an asymmetric design, with or without a hook structure 13 guarding the entrance is less likely to experience back flow directly into the narrow bead introduction channel 5.

### Solid Phase Extraction (SPE) On-Chip

As explained earlier, the present invention allows applications of microfluidic analysis systems to be extended. One such extension is facilitating SPE directly on-chip. Preconcentration is a valuable tool that can be used to enhance the sensitivity of microfluidic devices. To determine the ability of a packed SPE bed constructed on a microchip to preconcentrate an analyte, the inventors concentrated a 1.0 nM solution of BODIPY reagent from 50 mM phosphate buffer. Solution conditions utilized were similar to those used for protein and peptide analysis in HPLC-CE systems. [Bushey, M. M.; Jorgenson, J. W. *Anal. Chem.* 1990, 62, 978-984. Castagnola, M.; Cassiano, L.; Rabino, R.; Rossetti, D. V. *J. Chromatogr.* 1991, 572, 51-58.] The BODIPY reagent, when diluted in aqueous buffer, exhibits a high affinity for ODS material and is an excellent fluorophore. The preconcentration and elution of the BODIPY reagent was carried out in four steps: equilibration of the SPE bed with buffer; sample introduction; buffer flush; and elution of analyte. Following rinsing of the packed bed with phosphate buffer, a solution of 1.0 nM BODIPY was placed in main reservoir 1, and +200 V was applied for 2 minutes, with main reservoir 2 grounded. The EOF (0.2 mm/sec,  $1.2 \times 10^{-9}$  L/sec) flowed towards reservoir 2, carrying the BODIPY onto the SPE bed during the loading step. As shown in Figure 4A, fluorescence of the absorbed BODIPY occurred initially at the first few layers of beads 12 only (near the top of the Figure). Figure 4B shows the SPE bed after 1.5 minutes, with a total of  $1.4 \times 10^{-16}$  moles of BODIPY reagent loaded on the bed (assuming complete capture of the dye). No sample breakthrough was observed with BODIPY, due to its high affinity for the ODS material. In fact, visual observation indicated that after concentrating 1.0 nM BODIPY solution for two minutes only 5% of the physical volume of the SPE bed was utilized suggesting that the capacity of the 330 pL bed was about  $2.8 \times 10^{-15}$  moles of analyte. A buffer wash step was used after loading to wash sample remaining

within the channel 11 onto the bed (in chamber 4). The solutions in reservoirs 1 and 2 were then replaced with acetonitrile, and the dye was eluted with solvent moving in the same direction as the initial loading step (or by reversal of the potential gradient during the elution step, it could be directed back towards the original sample reservoir). Both procedures work well, but the latter was more convenient for our testing. Figure 5 shows graphically the 3-step preconcentration experiment for a 1.0 nM BODIPY sample following bed equilibration. The 90 second loading step showed an increase in signal as the fluorescent sample passed by the detector positioned as shown in Figure 1A. This was followed by a 60-second rinse step. Acetonitrile was then used to elute the BODIPY reagent off the bed in the opposite direction to which it was loaded, eliminating the need for detector repositioning. The BODIPY reagent eluted in a relatively narrow 3-second band following a 90-second preconcentration step exhibiting a many fold concentration increase compared to the original sample. The fluorescence of the BODIPY (1.0 nM) reagent was tested in both buffer and acetonitrile and did not show a significant difference in intensity for either of the solvents. The preconcentration factor (P.F.) can be estimated using equation (1):

$$P.F. = \frac{V_i}{V_f} = \frac{t_{pre} \bullet f_{buff}}{t_{elute} \bullet f_{elute}} \quad (1)$$

where  $V_i$  is the volume of buffer containing analyte and  $V_f$  is the volume of acetonitrile containing analyte. The volume  $V_i$  is the product of the preconcentration time ( $t_{pre}$ , sec.) and the electroosmotic flow of the sample being concentrated ( $f_{buff}$ , L/sec.) while  $V_f$  is the product of width of the eluted analyte peak ( $t_{elute}$ , sec.) and the flow rate of the eluting solvent ( $f_{elute}$ , L/sec). For this case, the analyte was preconcentrated by a factor of at least 100 times. After sufficient concentration the BODIPY is easily observed visually on the SPE bed.

Different sample loading times were utilized to increase the



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amount of preconcentration. In the experiments, preconcentration times ranging from 120 - 532 seconds were studied yielding preconcentration factors of 80 - 500. Peak area (rsd 3-11%) plotted versus preconcentration time yielded a linear relationship ( $r^2 = 0.9993$ ) over the studied conditions.

## 5 Capillary Electrochromatography (CEC) On-Chip

As explained earlier, another application facilitated by the present invention is on-chip capillary electrochromatography (CEC). Reversed phase mode CEC was performed on a chamber 4 packed with octadecyl silane beads 12 equilibrated with buffer. Due to the lack of an injector within the chip design, the samples were loaded onto the front of the chromatographic bed in 50 mM ammonium acetate buffer, pH 8.5 (see "Solutions and Reagents," above). Both compounds were totally retained under these conditions, as indicated by a lack of analyte signal in the loading and flush steps. The loading step functioned to both introduce the sample and preconcentrate the retained analytes at the front of the bed [Swartz, M. E.; Merion, M.; *J. Chromatogr.*, 1993, 632, 209-213.] Figure 6 shows the three steps involved in the CEC separation of BODIPY and fluorescein with a mobile phase composition of 30% acetonitrile/ 70% aqueous 50 mM ammonium acetate. Once the mixed mobile phase reaches the bed, both compounds begin to undergo chromatography and are eluted from the bed. The compounds are completely eluted and separated in less than 20 sec on less than 200  $\mu\text{m}$  of chromatographic bed, yielding a plate height of 2  $\mu\text{m}$  ( $N = 100$  plates or 500 000 plates/m) for the fluorescein peak. Under these conditions, the fluorescein is eluted prior to the BODIPY reagent. Peaks were identified by comparing retention times of the standards with those of the mixture. At pH 8.5 fluorescein possesses a net (-2) charge while BODIPY is neutral. In a normal CZE separation the electrophoretic mobility of fluorescein would oppose the EOF, causing the BODIPY to elute prior to fluorescein. In this case the elution order of the two components is reversed, indicating an interaction between the analytes and the stationary phase. The BODIPY being more

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hydrophobic has a higher affinity for the chromatographic material than does fluorescein causing the BODIPY to be retained more and eluted later.

Finally, Figures 7A-7D shows the CEC separation of BODIPY and fluorescein utilizing mobile phases with different concentrations of acetonitrile. It was observed that the increased acetonitrile concentration lowers the polarity of the mobile phase, decreasing the amount of time required for the BODIPY to elute. The elution time for fluorescein does not change, indicating little to no chromatographic retention except at low % acetonitrile. Decreasing the acetonitrile concentration provides baseline resolution, but leads to more extensive band broadening.

Our present results are comparable to that reported for open tubular CEC on a chip [Jacobson, S. C., Hergenröder, R., Koutny, L. B., Ramsey, J. M. *Anal. Chem.* 1994, 66, 2369-2373. Kutter, J. P.; Jacobson, S. C.; Matsubara, N.; Ramsey, J. M. *Anal. Chem.* 1998, 70, 3291-3297. He, B., Tait, N., Regnier, F. *Anal. Chem.* 1998, 70, 3790-3797.].

### **Immunoassay Using Bead-based Reagents**

Immunoassay on beads, or immunosorbent assays involves placing either an antibody or antigen on the surface of the bead. As a solution containing an antigen passes over the beads, the antigen specifically binds the antibody. In this way the specificity of the antigen for the antibody is utilized to separate it from other species in solution. Later the solution conditions are changed so that the antibody or antigen is eluted from the beads and is detected as either complex or the free antibody. The development of immunosorbent assays on chip is attractive because of the small amounts of reagents that are consumed. In addition microchips offer very fast analysis times compared to conventional methods performed in micro titer plates or in syringes packed with immuno-beads. Immunosorbent assays on-chip also provide lower concentration detection limits than solution phase immunoassays on-chip, making the development of bead based immunoassay on-chip important.

Beads that have specific enzymes linked to them are packed into the chamber created by the two weirs. The use of beads is preferential because of the increased surface area of the beads as opposed surface area of the channel walls. The higher surface area leads to a greater capacity and more efficient trapping of the analyte. The weirs form a well-defined chamber for the immunoassay beads to pack. The inventors have demonstrated bead-based immunoassay on chip for the enzyme theophylline. In the experiment magnetic beads coated with protein A are packed within the chamber of the chip. Later the antibody (antitheophylline) is flowed across the bed in a 1 mM tricine buffer pH 8.0. When the antitheophylline flows through the packed bed the antibody binds to the protein A. The antitheophylline was passed over the bed for several minute to ensure that the bed is saturated with antibody. A buffer washing step was then utilized to remove the remaining unbound antibody from the chamber and channels. The bed was then saturated with fluorescently labelled theophylline (diluted from a kit) by flowing it through the bed where it binds to the antitheophylline. The point at which the bed was saturated was determined by monitoring fluorescence below the bed and determining the point where the breakthrough curve (Figure 1) plateaus. Following breakthrough the theophylline solution is washed from the device using a buffer flush step. A chaotropic agent is then added to elute the theophylline from the bed as either free protein or theophylline/antibody complex. Chaotropic agents can be of various types, however in this example a mixture of 90% acetone/ 10 % tricine buffer was used. Once the chaotropic agent reaches the packed bed the theophylline is eluted in a relatively narrow band (Figure 2).

Although normally under these circumstances a competitive assay would be performed the direct assay demonstrates the ability of the chamber formed by the weirs to act as an immunoassay bed.

### 30 Enzyme Reactor Beds

There have been several methods developed for

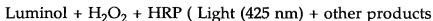
immobilizing enzymes onto solid supports like beads. Once immobilized the enzyme beads can be packed into beds to perform chemical reactions on solutions as they are flowed through them. Normally a solution containing a substrate is passed through the bed. When the substrate comes in contact with the enzyme the enzyme reacts with the substrate to yield a product. The product resulting from the reaction of the immobilized enzyme and substrate can be later used as a method of detection or in other synthetic processes. This example illustrates the use of the immobilized enzyme horse radish peroxidase(HRP) and xanthine oxidase(XO) on porous silica beads (5  $\mu\text{m}$  diameter). These results show that enzymes, once immobilized onto beads, can be trapped/packed into the weir device, where they are still active and can be used as an enzyme reactor bed.

XOD and HRP were immobilized onto Nucleosil 1000-5 silica beads (Machrey-Nagel, Germany) that had been silanized with 3-aminopropyltriethoxysilane, by crosslinking with gluteraldehyde (Sigma). The immobilization of enzymes on glass beads has been described previously and is known by practitioners of the art. All studies were performed using 50 mM boric acid adjusted with 1 M NaOH to pH 9.

The immobilization of HRP and XOD was performed to demonstrate two principals. First was the ability to pack the enzyme immobilized beads within the weir device and then second was to demonstrate that the enzyme was still active and could be utilized to catalyze reactions once packed. To show each of these principals a chemiluminescent reaction was performed using the weir device.

The ability to pack immobilized enzymes allows different methods of detection to be used for certain analytes. For example the luminol chemiluminescence (CL) reaction can be used for very sensitive determinations when only small amounts of analyte are available or when labeling reactions are otherwise difficult to perform. CL reactions are unique in that they do not require a light source simplifying the detection scheme. The chemiluminescence reaction catalyzed by HRP is

shown below.



Beads immobilized with HRP were packed into the weir device and a solution containing the reagents for the reaction passed through the bed.

5 The immobilized HRP was found to catalyze the chemiluminescent reaction when a solution of  $\text{H}_2\text{O}_2$  (100 (M) and luminol (10 mM) was flowed over a bed that had been packed with beads containing immobilized HRP. Light generated from the reaction was detected downstream from the enzyme bed.

10 However, it was noticed that with each successive trial the light generated from the CL reaction was lower than in the previous trial Figure 1. This is probably caused by a decrease in the activity of the enzyme with each successive run. These results evidence the advantage of a method of removing the exhausted beads and replacing them with fresh ones, such as  
15 discussed for the replacement of ODS beads within the weir device.

### Alternative Embodiments

While a two weir embodiment of the design according to the present invention has been described above, other embodiments are also possible. For example, it is possible to implement a single weir design to  
20 form an on-chip reactor bead (i.e. not having a second weir 6 located upstream in the main channel 11). Specifically, by providing a downstream weir 7 formed across the main channel and providing pressure only in a downstream direction (i.e. from main reservoir 2 and side channel 3 to main reservoir 1), it has been observed that packing can  
25 be achieved against the downstream weir 7. However, it is noted that a single weir design may result in the formation of a ragged leading edge for the packed bed that reduces separation efficiency when used for SPE or CEC. Additionally, the high back pressure associated with a long bed of small beads limited the length of the pack to about 4 – 6 mm. A high

pressure fitting for the microchip would allow high pressure pumping and allow somewhat greater lengths.

Other types of forces may also be used to create a packed bed using a single weir design. For example, it was also possible to achieve a limited  
5 degree of packing (to a length of a few millimeters) using electrokinetic forces, directed only in a downstream direction (i.e. from main reservoir 2 and packing material reservoir 3, to main reservoir 1).

In addition to varying the number of weirs, it is also possible to provide more than one inlet or outlet to a chamber, as shown in  
10 alternative embodiments of the present invention in Figures 8A-8C.

In Figure 8A, a chamber 4 is formed between two weirs 6, 7. Two side channels 5a, 5b are provided to serve as an inlet or outlet to the chamber 4. As shown in Figure 6, the side channels 5a, 5b may be offset relative to each other to better facilitate packing of the chamber. A second  
15 side channel is added to allow the beads to be flushed out to waste at the other end of the trapping zone, or to allow the flushing agent to be delivered from an alternate reservoir. The latter design can prevent used beads from contaminating the fresh bead stream, and/or prevent sample and sample waste solutions from being directed into the trapping zone  
20 during flushing.

As shown in Figure 8B the side channel in this design may have one or more optional branches 5c, to allow the side channel 5b to be flushed of beads, or to allow beads being flushed out of the trapping zone to be directed, for example, into a waste reservoir instead of into the  
25 packing material reservoir 3 (not shown).

Another embodiment is shown in Figure 8C, in which a side channel weir 16 is provided near the entrance of a third side channel 5d to the chamber 4, to allow fluid flow without passage of beads. This "weired" side channel 5d may be used, for example, to release pressure build up in  
30 the chamber 4 during loading of the beads, particularly when the length of the chamber 4 (as measured between the weirs 6, 7) is greater than 4-6 mm.

In all three embodiments shown in Figures 8A-8C, the side channel

entrance into the chamber 4 may be modified to include a hook or similar shape, as described earlier, in order to prevent direct "line-of-sight" flow from a side channel into the chamber 4, or vice versa. As explained earlier, this entrance modification serves to spray the beads into the trapping zone in order to assist packing, and to reduce the tendency of the beads to exit from the chamber 4 during later use.

Loading of beads with more than one side channel, as shown in Figures 8A-8C, is performed in a manner similar to that for a single side channel, two weir design, (as described above) except that a potential must also be applied to the additional side channels to prevent flow into those side channels when using electrokinetic loading. During removal of the beads a voltage may be applied to a second side channel (e.g. side channel 5b in Figure 8A) to drive beads out of the trapping zone or chamber 4, applying voltage potentials such as those used with the single side channel design but adjusted for the potential drop in the additional side channel. As will be appreciated, the direction of flow during the flushing step can be controlled by the polarity of the applied voltage.

When using pressure driven flow to load beads, a back pressure must be applied to the additional side channels during loading, or else the reservoirs attached to the additional side channels may be temporarily sealed. When flushing the beads from the chamber 4, a pressure may be applied to the bead supply channel 5a to flush beads out of one or more additional side channels.

When performing SPE or CEC using a multiple side channel design and electrokinetic forces, a voltage may be applied to the additional side channels to prevent leakage of sample or beads out of the trapping zone and into the side channels, substantially in the same manner as described for a single side channel in the trapping zone. When using pressure driven pumping, the side channels may have enough positive pressure applied to eliminate flow into the side channel, or else the reservoirs attached to the respective side channels can be temporarily sealed.

### Immobilizing the packed ODS beads with polymer

After packing the chromatographic bed with beads using electrokinetic, magnetic or pressure packing techniques, a reasonable portion of the bead introduction channel was then packed with the beads using one of these packing techniques. Excess beads in the reservoir were then removed. Then about 40 ml of a monomer mixture was pipeted into the bead reservoir and delivered by pressure or electrokinetic flow along the bead introduction channel, towards the beads. During this step, a change in refractive index in a region within the bead introduction channel was used to monitor the position of the monomer solution, and flow was stopped well before the monomer reached the packed bead bed.

A typical monomer solution may be prepared as follows: to a vial that contained 800 ml of a ternary solvent mixture that contained 10 wt% H<sub>2</sub>O, 40 wt% 1,4-butanediol and 50 wt% 1-propanol, 200 ml of a mixture of 2,2'-azobisisobutyronitrile (AIBN, 2 wt%) and ethylene dimethacrylate (EDMA) was added. (C. Peters et al, Anal. Chem. 1997, 69, 3646-3649.) This monomer solution was then purged with N<sub>2</sub> for 15 min to remove dissolved oxygen. Other polymerizable solution of monomer may also be used.

The device was then kept in an oven at 60 °C for 24 - 48 hr. The device was taken out from the oven and cooled down to room temperature, with all reservoirs covered to prevent evaporation. Alternatively, photo-initiated polymerization with AIBN or other initiator may be used to polymerize the monomer solution, without a need for extended heating of the device. Following polymerization the device was rinsed with acetonitrile then with buffer. Mobile phase compositions of up to 100% acetonitrile could be used in such devices without destabilizing the bead bed.

### Dimension Guidelines

While the theoretical limits of various dimensions of a microfluidic device designed according to the present invention are not



known, the inventors have adopted some general guidelines for practical purposes, which are discussed below.

It is thought that the length of the trapping zone may range anywhere from about 10  $\mu\text{m}$  up to about 200  $\mu\text{m}$  (using a coiled or serpentine path if necessary to allow for incorporation of such a length within the confines of a single device wafer). The trapping zone length required will be dependent upon the application and will also be limited by the forces which may be applied to achieve packing and unpacking. For example, on-chip CEC would require relatively long trapping zones, with a preferred upper limit of about 5 cm.

As to the depth of the trapping zone, sample and waste channels, a practical range is estimated to be about 400  $\mu\text{m}$  to 0.25  $\mu\text{m}$ . More preferably, the upper limit should be about 100  $\mu\text{m}$  and the lower limit should be about 10 % larger than the particle depth at a minimum.

Also, in order to reduce the likelihood of clogging, the bead delivery and bead waste channels (side channels 5, 5a-5d) preferably should be at least about 3 times deeper and three times wider than the bead diameter.

The maximum dimensions of the side channels 5, 5a-5d are also dependant upon the relative flow resistances required (i.e. the flow resistance of the side channel versus the main channel and the weirs, so as to minimize side channel backflow during use). Generally speaking, the flow resistance of the side channels should be higher than the flow resistance of weirs to minimize the backflow problem.

The accompanying tables provide information on the calculated effect of channel and weir dimensions on the volumetric flow rates out of the trapping zone, as a function of flow channel depth, weir depth and side channel length using pressure driven flow.

In the tables below, what is called channel W is element 1 in Figure 1A; what is called channel C is called element 5 in Fig 1A; and what is called channel C' is element 3 in Fig 1A

Correlation to Fig 1A		Width	Length
	<u>20 <math>\mu</math>m Deep</u>		
Element 1	Channel W	600	6,500
	Weir	280	variable
Element 5	Channel C	50	variable
Element 3	Channel C'	600	3,500
	<u>10 <math>\mu</math>m Deep</u>		
Element 1	Channel W	580	6,500
	Weir	280	variable

The volumetric flow rates were estimated using the Navier-Stokes equation for a rectangular channel cross section and Perry's tabulated values of the effect of channel shape. The flow resistance of a channel with half width  $a$  and half depth  $b$  is given by equation 2:

$$\Delta P/U = hL/abN \quad (2)$$

where  $\Delta P$  is the pressure drop along a channel segment of length  $L$ ,  $U$  is the average linear flow velocity,  $h$  is the viscosity, and  $N$  is a form factor dependent upon the cross sectional ratio  $b/a$  ( $b < a$ ). The factor  $N$  may be estimated from solutions to the Navier-Stokes equation for pressure driven, parabolic flow, and was tabulated by Perry in Chemical Engineer's Handbook, (3rd edition, 1950) pp 387. The goal in device design is to make the resistance of the side channel,  $C$  in the Tables, higher than the resistance of the weir and the following flow channel  $W$ , so that flow across the weir is favoured. When flow elements are in series the fluid resistance given by the right hand side of equation 1 for each segment can

- be added in the manner that the resistance of series electrical impedances can be added. When fluid elements are in parallel the inverse of their fluid resistance can be added to obtain the inverse of the total impedance, as is done for parallel electrical resistances. The volumetric flow rate,  $Q$ , through a channel or a combination of channels is then given by equation 3.

$$Q = ab\Delta P/R_f \quad (3)$$

- Where  $R_f$  is the resistance to fluid flow defined by the right hand side of equation 1, combined together for all channel segments as discussed above.
- 10 The ratio,  $r$ , of volumetric flow rate across the weir,  $Q_w$  versus into the side channel,  $Q_C$ ,  $r = Q_w/Q_C$ , should be large to ensure the percent of solution flowing across the weir,  $\%Q_w = 1/(1+r)$ , is high. This can be accomplished by using a long narrow side channel compared to a wide main channel, by increasing the depth of the weir relative to the depth of
- 15 the other channels, by decreasing the depth of the side channel relative to the main channel, etc, as indicated by several calculations presented in the Tables.

**Table: Volumetric Flow Ratios for 10 and 20 $\mu$ m Deep Designs**

Channel depth	Channel C Length	Weir Depth	Weir Length	Volumetric ratio $r$	$\% Q_w$
20 $\mu$ m	15,000	3	20	12.58	92.6
	25,000			20.85	95.4
	15,000		10	18.75	94.9
20 $\mu$ m	15,000	1	20	0.687	41.1
	25,000			1.16	53.6
	15,000		10	1.37	57.8

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10 $\mu$ m	15,000	3	40	38.9	97.5
	25,000			63.7	98.5
	15,000		30	41.7	97.7
10 $\mu$ m	15,000	1	40	4.14	80.5
	25,000			6.87	87.3
	15,000		30	5.83	84.3

**Table: Fixed Device Dimensions for Calculations with a Given Etch Depth**

	Width	Length
<u>20 <math>\mu</math>m Deep</u>		
Channel W	600	6,500
Weir	280	variable
Channel C	50	variable
Channel C'	600	3,500
<u>10 <math>\mu</math>m Deep</u>		
Channel W	580	6,500
Weir	280	variable
Channel C	30	variable
Channel C'	580	3,500

**Integrated Analytical Procedures**

It will be appreciated that the various features of the present invention as described above may be utilized in a more complex microfluidic design.

Figure 9 shows a multiple weir and multiple side channel design, generally referred to by reference numeral 20, in which several trapping zones are integrated, each serving a different function.

As an illustrative example, in a first trapping zone 25, formed between weirs 6a and 6b, beads loaded with an antibody to a specific protein are introduced via side channel 25 (and exit via side channel 26). A cell lysate or serum sample or other protein source is directed from a sample reservoir (not shown) and loaded into the chip via sample inlet 21 and entrance channel 38 (the sample is removed at sample outlet 22 and an eluent inlet 23 is also provided at the entrance channel. The sample is then passed into the antibody bead bed in trapping zone 25 to isolate a specific protein, while the effluent is directed towards waste outlet 27.

A chaotropic elution agent, such as an acetonitrile, water mix, is then introduced (eluent inlet 23) to elute the protein from the column and deliver it to the next trapping zone 30 (formed between weirs 6c and 6d) where it is digested by a protease enzyme immobilized on beads loaded into the zone 30 (via side channels 29, 31). The effluent at this stage would be directed towards waste outlet 32. After sufficient reaction time, a buffer is delivered (elution inlet 28, running buffer 28a, waste from bed 25) to flush the protein digest from the bed and into the next trapping zone 35 (formed between weirs 6e and 6f) with effluent delivered to waste outlet 39.

The third trapping zone 35 contains a solid phase extraction material (packed and unpacked via side channels 34, 35), allowing concentration of the digest peptides onto the bed in zone 35. An elution solvent, such as a methanol/aqueous mixture or acetonitrile/ aqueous mixture is then introduced (elution inlet 33, running buffer 33a) to deliver (exit channel 37, waste 39, or collection 40) a concentrated protein digest to another location on the chip for final analysis.

### **Packed Bed Chip to Electrospray Mass Spectrometry Interface**

Packed bed flow channels according to the present invention

may be interfaced to a mass spectrometer via an electrospray coupler 41, as illustrated in Figure 10. The packed bed 4 may perform an enzyme digestion of a protein, affinity purification and pre-concentration of a specific chemical or protein, solid phase extraction concentration enhancement, or capillary electrochromatographic separation, or any combination of these and other steps, prior to electrospray introduction in to a mass spectrometer. The chip to electrospray interface may be made using any method that provides a less than 100 nL dead volume, preferably less than 1 nL and most preferably less than 100 pL dead volume at the coupling region. A method such as that described by Wang et al., or Karger can be used to create the interface [Bings, N.H.; Wang, C.; Skinner, C.D.; Colyer, C.L.; Thibeault, P.; Harrison, D.J. *Anal. Chem.* 71 (1999) 3292-3296. Zhang, B.; Liu, H.; Karger, B.L.; Foret, F. *Anal. Chem* 71 (1999) 3258-3264].

While the present invention has been described by reference to various preferred embodiments, it will be understood that obvious changes may be made and equivalents substituted without departing from the true spirit and scope of the invention which is set out in the following claims.

**WE CLAIM:**

1. A method of treating a sample within a microfluidic analysis system, comprising the steps of:
  - 5 a) providing a main channel having a trapping zone suitable for trapping packing material;
  - b) providing a slurry of a reagent treated packing material prepared in a solution having a predetermined composition of a solvent;
  - 10 c) inducing a flow of said packing material into said trapping zone through a flow channel connected to said trapping zone so as to load said trapping zone and form a packed bed of said packing material;
  - 15 d) flowing a sample containing analytes through said packed bed, said reagent treating the sample, whereby the sample leaving the trapping zone has an altered analyte composition.
2. The method claimed in claim 1, further comprising the step of:
  - 20 e) adjusting the composition of the solvent, so as to affect the aggregation of said packing material and the stabilization of the packed bed.
3. The method claimed in claim 1 or 2, wherein, step b) comprises providing packing material comprising porous beads.
4. The method claimed in claim 3, wherein said porous beads are selected to have a diameter in the range from about 0.7 to about 10.0  $\mu\text{m}$ .
- 25 5. The method claimed in claim 3, wherein said porous beads are selected to have a diameter in the range from about 1.5 to about 4.0  $\mu\text{m}$ .

acetonitrile, and step e) comprises adjusting the concentration level to less than about 50% to stabilize the packed bed.

7. The method claimed in claim 4 or 5, wherein said solvent is acetonitrile, and step e) comprises adjusting the concentration level to less than about 30% to stabilize the packed bed.

8. The method claimed in claim 6 or 7, further including the steps of adjusting the concentration level to above 50% to destabilize the packed bed, and reversing the flow in step c) so as to unload said trapping zone.

9. The method claimed in claim 8, further including the step of repeating step c) so as to reload said trapping zone, and readjusting the concentration level to restabilize the packed bed.

10. The method claimed in claim 1, further comprising the steps of:  
before step c), adding a neutral surfactant to said packing material so as to inhibit aggregation; and  
after step c), removing the neutral surfactant to promote aggregation.

11. The method claimed in claim 1, further comprising the steps of:  
after step c) introducing a polymerizable agent into the flow channel and polymerizing said agent, so as to stabilize the packed bed.

12. The method claimed in claim 1, wherein step d) comprises applying a fluid force to induce the flow of said packing material.

13. The method claimed in claim 1, wherein said packing material



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comprises at least some electrically charged particles and step d) comprises applying a voltage potential to induce the flow of said packing material.

14. The method claimed in claim 1, wherein said packing material comprises at least some particles susceptible to a magnetic field and step d) comprises applying a magnetic field to induce the flow of said packing material.

15. The method claimed in any one of claims 1-14, including the step of providing a hook structure at the connection point between said flow channel and said trapping zone, so as to prevent direct line-of-sight entry of said packing material, thereby to promote even packing.

16. A method of trapping bead based reagents within a microfluidic analysis system, comprising the steps of:

- a) providing a main channel having a trapping zone suitable for trapping bead based packing material;
- 15 b) providing a slurry of a reagent treated beads prepared in a solution having a predetermined composition of a solvent;
- c) inducing a flow of said beads into said trapping zone through a flow channel connected to said trapping zone so as to load said trapping zone and form a packed bed of said beads; and
- 20 d) adjusting the composition of the solvent, so as to affect the aggregation of said packing material and the stabilization of the packed bed.

25 17. A microfluidic analysis system, comprising:

- a) a substantially planar substrate having an upper surface;
- b) at least one main channel formed into said upper surface,

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said main channel having first and second ends and a defined direction of flow in use;

- c) a cover plate arranged over said planar substrate, said cover plate closing off said channel from above; and
- 5 d) a first weir formed across said main channel and between said first and second ends of said channel, said first weir providing at least one flow gap to allow, in use, at least some fluid to flow past said first weir while trapping packing material having constituent particles that are
- 10 generally larger than said flow gap.

18. The microfluidic analysis system claimed in claim 17, further comprising at least one side channel formed into the upper surface of said planar substrate, said side channel being connected at a first end to said main channel at a location upstream from said first weir, and at a second

15 end to a reservoir, said side channel providing a higher flow resistance than said main channel.

19. The microfluidic analysis system claimed in claim 18, further comprising a second weir located upstream from said connected first end of said side channel, said first and second weirs forming a chamber

20 therebetween, said second weir providing at least one flow gap to allow, in use, at least some fluid to flow past said second weir while trapping said packing material within said chamber.

20. The microfluidic analysis system claimed in claim 19, wherein, each side channel connection to said main channel is provided with a

25 hook structure curved to one side whereby, in use, packing material is sprayed into said chamber to facilitate even packing.

21. The microfluidic analysis system claimed in claim 20, wherein, said hook structure at least partially obstructs direct line-of-sight entry of

packing material from said side channel into said chamber and forms a chamber mouth to one side of said hook structure.

22. The microfluidic analysis system claimed in any one of claims 17-19, wherein, said flow gaps comprise a generally uniform gap between  
5 said cover plate and the top of said weirs.

23. The microfluidic analysis system claimed in any one of claims 17-19, wherein, said flow gaps comprise a plurality of substantially vertical gaps in said weirs.

24. The microfluidic analysis system claimed in any previous  
10 claim, wherein, said system is formed entirely on a single microfluidic chip.

25. A method of creating a packed reactor bed in the microfluidic analysis system claimed in claim 18, said method comprising, providing a non-conductive substrate and effecting an electrokinetic flow by applying a  
15 relatively high voltage at said second end of said main channel and at said reservoir, said reservoir containing packing material, and providing a relatively low voltage at said first end of said main channel, so that packing material flows from said reservoir into said main channel and is trapped against said first weir.

20 26. The method as claimed in claim 25, wherein, packing material is removed from said main channel by providing a relatively high voltage at said first and second ends of said main channel while providing a relatively low voltage at said reservoir.

27. A method of creating a packed reactor bed in the microfluidic  
25 analysis system claimed in claim 18, said method comprising, effecting a pressure driven flow by providing a relatively high pressure at said second

end of said main channel and at said reservoir, said reservoir containing packing material, and providing a relatively low pressure at said first end of said main channel, so that packing material flows from said reservoir into said main channel and is trapped against said first weir.

- 5     28.         The method as claimed in claim 27, wherein, packing material is removed from the said main channel by providing relatively high pressure at said first and second ends of said main channel while providing relatively low pressure at said reservoir.

29.         A method of packing the chamber in the microfluidic analysis  
10     system claimed in claim 3, said method comprising, providing a non-conductive substrate and effecting an electrokinetic flow by applying a relatively high voltage at said reservoir, said reservoir containing packing material, and providing relatively low voltages at said first and second ends of said main channel, so that packing material flows from said  
15     reservoir into said chamber and is trapped by said first and second weirs.

30.         The method as claimed in claim 29, wherein, packing material is removed from the chamber by reversing said electrokinetic flow.

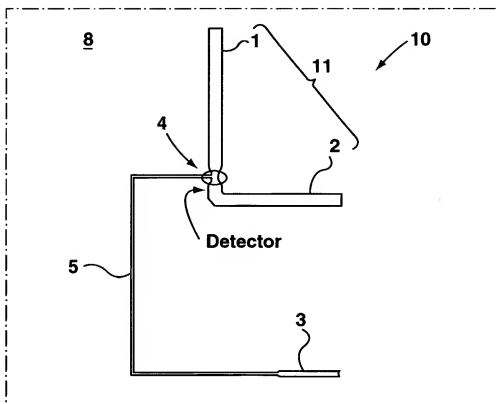
31.         A method of packing the chamber in the microfluidic analysis  
20     system claimed in claim 3, said method comprising, effecting a pressure driven flow by providing a relatively high pressure at said reservoir, said reservoir containing packing material, and providing relatively low pressure at said first and second main reservoirs, whereby, packing material flows from said packing material reservoir into said chamber and is trapped by said first and second weirs.

- 25     32.         The method as claimed in claim 31, wherein, packing material may be removed from the chamber by reversing said pressure driven flow.

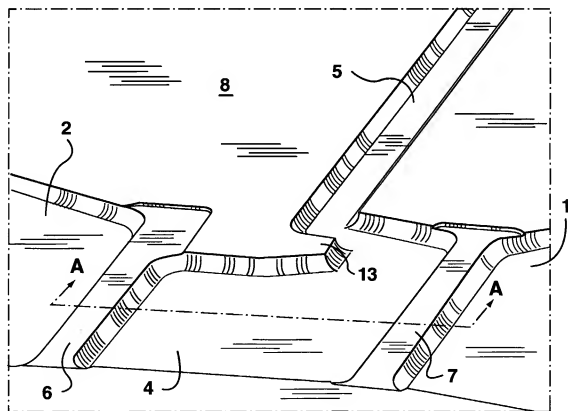
- 44 -

33. A method of packing the chamber in the microfluidic analysis system claimed in claim 32, said method comprising, providing magnetically charged packing material, and effecting a magnetically driven flow by providing a magnetically attractive force in the chamber, whereby,  
5 the packing material enters the chamber and is trapped by said first and second weirs.
34. The method as claimed in claim 34, wherein, packing material may be removed from the chamber by reversing said magnetic force in said chamber.
- 10 35. The method claimed in any one of claims 25-34, wherein, said packing material comprises porous beads.
36. The method claimed in claim 35, wherein, said beads are generally spheroid.
37. The method claimed in claim 36, wherein, said beads are  
15 initially suspended in a buffer solution.
38. The method claimed in claim 5, wherein, said buffer solution is an organic solvent miscible with water.
39. The method claimed in claim 38, wherein, said organic solvent is acetonitrile with a concentration level of up to 50%.

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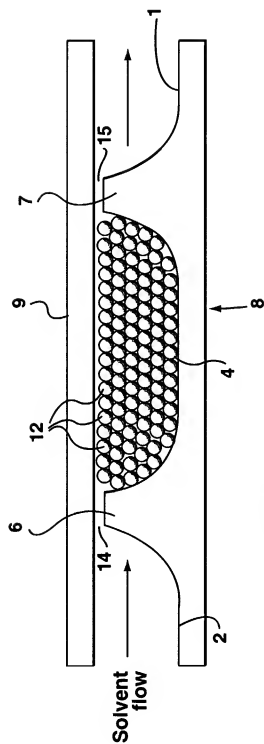


**FIG. 1A**

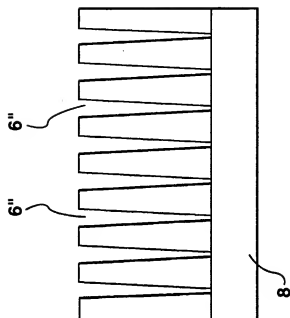
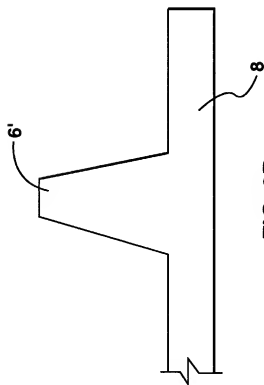


**FIG. 1B**

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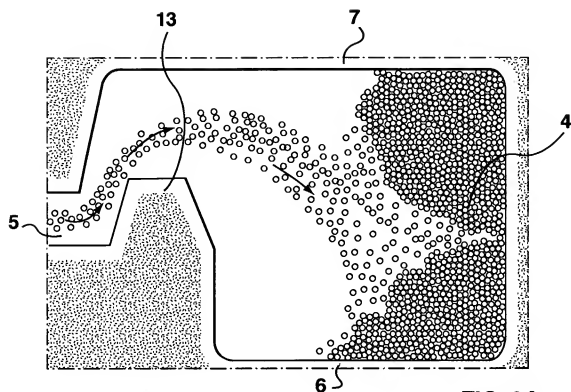
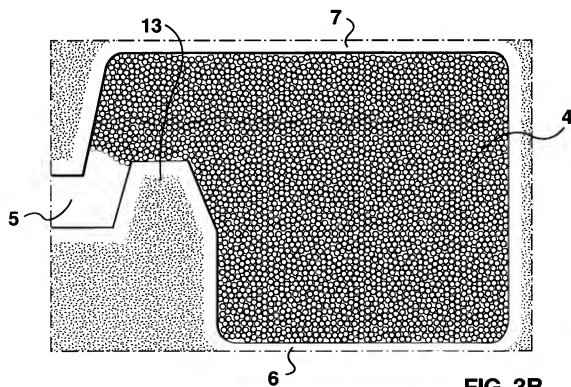


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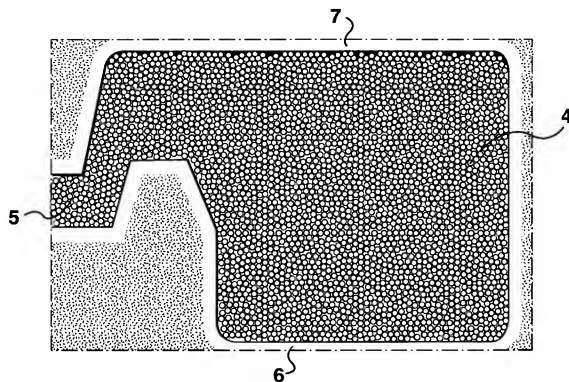
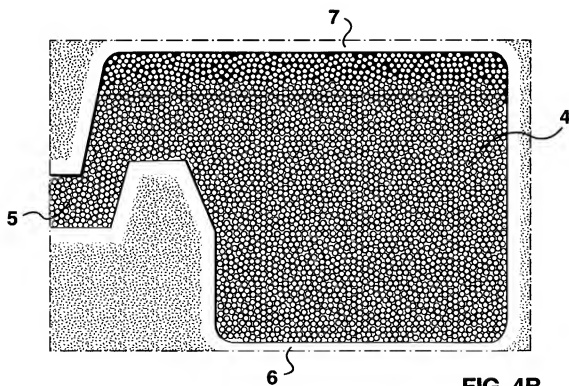
FIG. 2CFIG. 2B



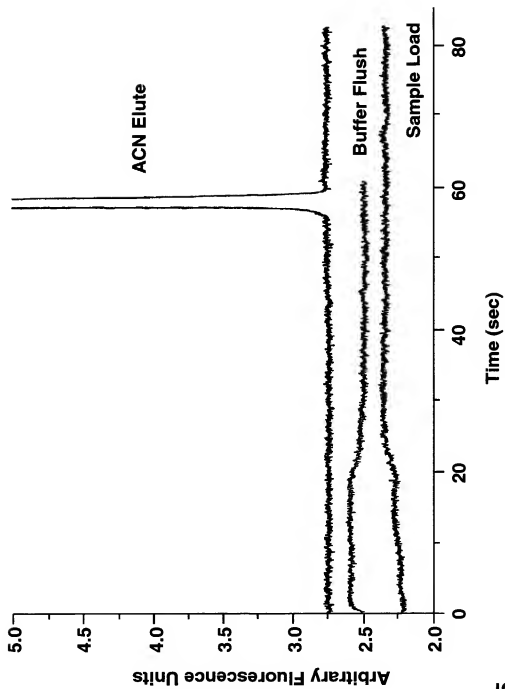
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**FIG. 3A****FIG. 3B**

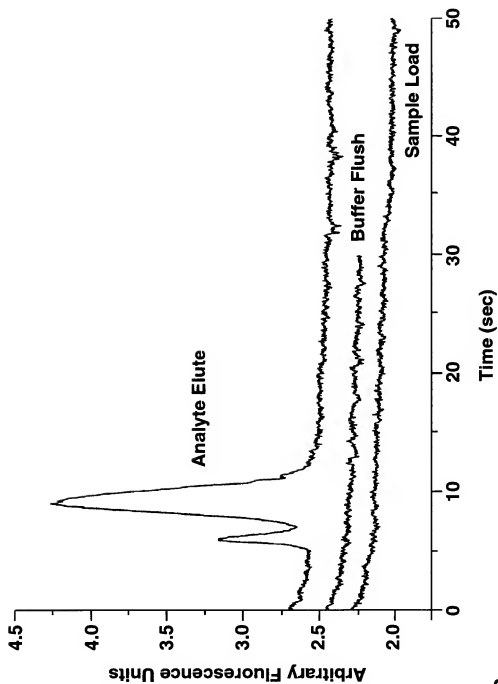
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**FIG. 4A****FIG. 4B**

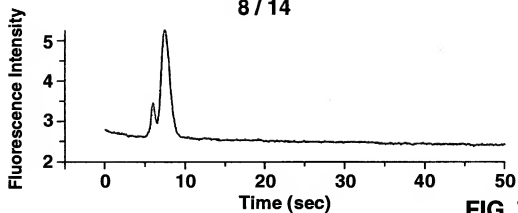
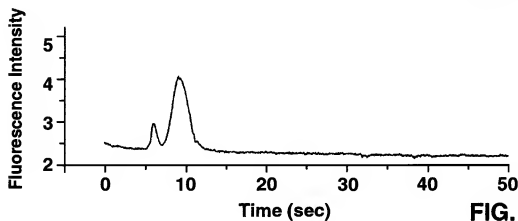
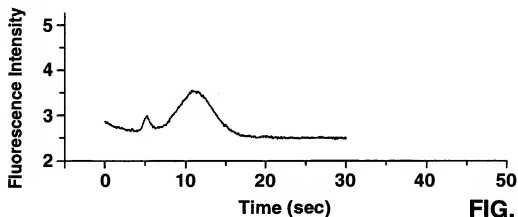
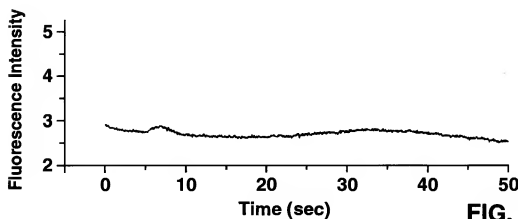
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**FIG. 5**

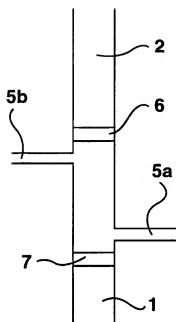
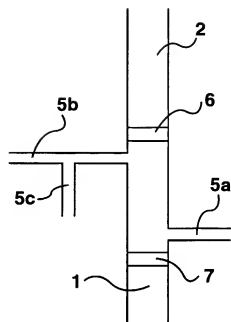
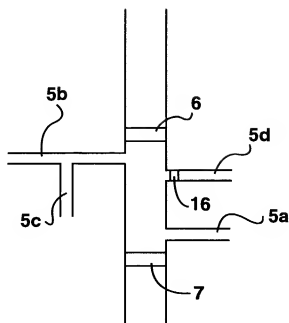
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**FIG. 6**

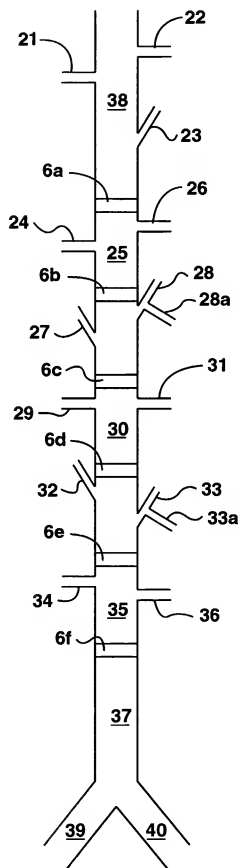
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**FIG. 7A****FIG. 7B****FIG. 7C****FIG. 7D**

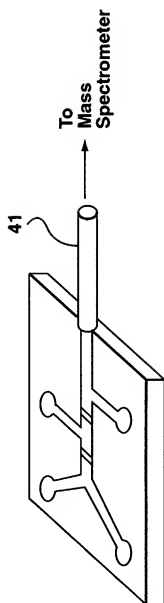
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**FIG. 8A****FIG. 8B****FIG. 8C**

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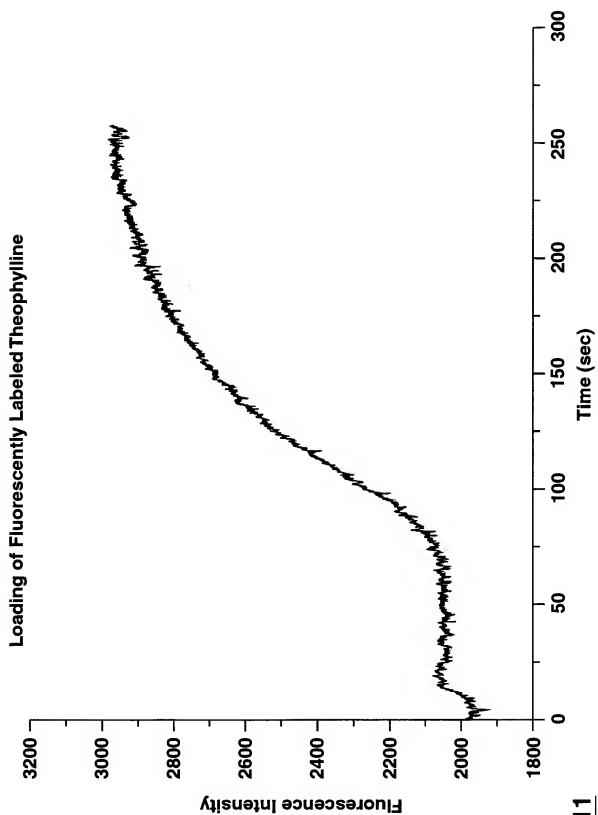
**FIG. 9**

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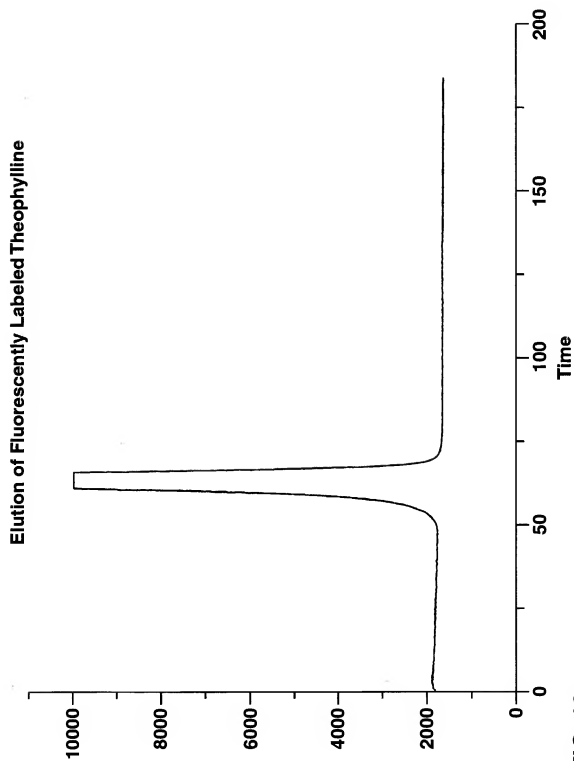
**FIG. 10**



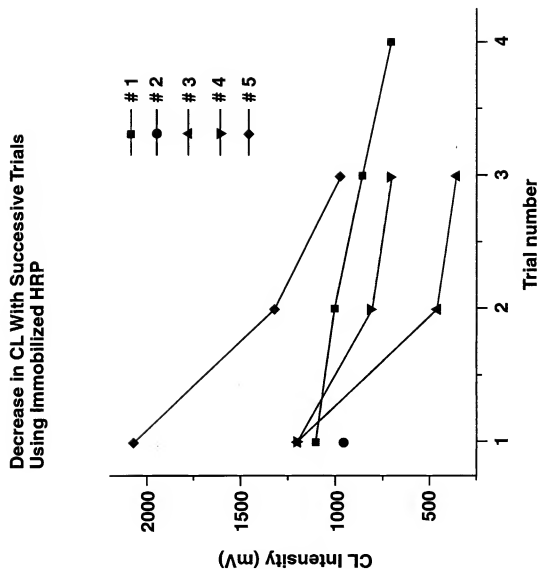
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**FIG. 11**

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**FIG. 12**

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**FIG. 13**

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 G01N27/447 G01N1/40 G01N30/56

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N 801J

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EP0-Internal

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 99 40174 A (ACLARA BIOSCIENCES INC) 12 August 1999 (1999-08-12) page 4, line 5 -page 5, line 33 page 8, line 17-22 page 24, line 38-41	1,12,13
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Y	US 5 453 163 A (YAN CHAO) 26 September 1995 (1995-09-26) cited in the application column 3, line 2 -column 5, line 23; figure 1	1,12,13
A	---	4,5,16
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☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

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\*O\* document referring to an oral disclosure, use, exhibition or other means

\*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search

22 March 2001

Date of mailing of the international search report

05/04/2001

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Brison, O

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>WRIGHT P B ET AL: "BEHAVIOR AND USE OF NONAQUEOUS MEDIA WITHOUT SUPPORTING ELECTROLYTEIN CAPILLARY ELECTROPHORESIS AND CAPILLARY ELECTROCHROMATOGRAPHY" ANALYTICAL CHEMISTRY,US,AMERICAN CHEMICAL SOCIETY. COLUMBUS, vol. 69, no. 16, 15 August 1997 (1997-08-15), pages 3251-3259, XP000699506 ISSN: 0003-2700 cited in the application abstract page 3253, right-hand column</p>	1,7
A	<p>EP 0 459 241 A (MILLIPORE CORP) 4 December 1991 (1991-12-04) column 2, line 26 -column 3, line 41 column 5, line 36 -column 9, line 32</p>	1
A	<p>WO 96 04547 A (LOCKHEED MARTIN ENERGY SYS INC ;RAMSEY J MICHAEL (US)) 15 February 1996 (1996-02-15) page 8, line 3 -page 11, line 8 page 24, line 8-33</p>	1,17
X,P	<p>CHIEM N ET AL: "Room temperature bonding of micromachined glass devices for capillary electrophoresis" SENSORS AND ACTUATORS B,CH,ELSEVIER SEQUOIA S.A., LAUSANNE, vol. 63, no. 3, May 2000 (2000-05), pages 147-152, XP004198333 ISSN: 0925-4005 the whole document</p>	17
A,P	<p>C. ERICSON ET AL.: ANALYTICAL CHEMISTRY., vol. 72, no. 1, 1 January 2000 (2000-01-01), pages 81-87, XP002163649 AMERICAN CHEMICAL SOCIETY. COLUMBUS., US ISSN: 0003-2700 cited in the application page 81</p>	1,16
A	<p>US 5 908 552 A (ZIMMERMANN HANS-PETER ET AL) 1 June 1999 (1999-06-01) column 1, line 44-58; figure 1 column 4, line 16-33</p>	1,16

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	CHIAM N H ET AL: "MICROCHIP SYSTEMS FOR IMMUNOASSAY: AN INTEGRATED IMMUNOREACTOR WITH ELECTROPHORETIC SEPARATION FOR SERUM THEOPHYLLINE DETERMINATION" CLINICAL CHEMISTRY,AMERICAN ASSOCIATION FOR CLINICAL CHEMISTRY. WINSTON,US, vol. 44, no. 3, 1998, pages 591-598, XP000937658 ISSN: 0009-9147 page 591 -page 592	17

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			US 6033546 A	07-03-2000
			US 5858195 A	12-01-1999
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